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The effect of thymidine kinase transduction and ganciclovir therapy on tumor vasculature and growth of 9L gliomas in rats

ZVI RAM, M.D., STUART WALBRIDGE, THOMAS SHAWKER, M.D., KENNETH W. CULVER, M.D., R. MICHAEL BLAESE, M.D., AND EDWARD H. OLDFIELD, M.D.

Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke; Department of Radiology, Clinical Center; and Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

Eradication of malignant brain tumors by *in situ* intratumoral, retrovirally mediated transfer of the herpes simplex virus thymidine kinase (HSVtk) gene, which sensitizes the tumor cells to ganciclovir, has recently been demonstrated in animal models. The observation that tumors studied *in vitro* and in animals can be completely eliminated despite only partial transduction of the tumor suggests a bystander mechanism that affects nontransduced tumor cells. Such a bystander effect is not completely understood and may represent a combination of several factors that lead to tumor eradication. Endothelial cells of the tumor blood vessels were shown to occasionally integrate the retroviral vector and thus become sensitized to ganciclovir. In the presence of vector-producer cells, which continuously release infectious viral particles, diffuse multifocal hemorrhages occurred during ganciclovir administration. When the tumor was composed of cells that had been transduced with the thymidine kinase gene before inoculation, no infectious viral particles were present within the tumor, no transduction of endothelial cells occurred, and no hemorrhages were observed during ganciclovir therapy. These observations suggest that tumor regression may be due, in part, to destruction of *in vivo* HSVtk-transduced endothelial cells after exposure to ganciclovir, resulting in tumor ischemia as one possible bystander mechanism.

The authors investigated this hypothesis using the subcutaneous 9L gliosarcoma tumor model in Fischer rats. The tumors were evaluated with Doppler color-flow and ultrasound imaging during the various phases of the study. Twenty rats received intratumoral injections of HSVtk retroviral vector-producer cells (6×10^7 cells/ml) 21 days after bilateral flank tumor inoculation. Ten rats were subsequently treated with intraperitoneal ganciclovir (15 mg/kg/ml twice a day) for 14 days starting on Day 7 after producer cell injection; 10 control rats received intraperitoneal saline injections (1 ml twice a day) instead of ganciclovir. Ultrasound and flow images were obtained before cell injection, before and during ganciclovir or saline administration, and after cessation of treatment. The number, location, and ultrasonographic appearance of tumor vessels and the tumor volumes were recorded.

The number of blood vessels in the tumors increased over time in both groups before treatment. Intratumoral cell injection without ganciclovir administration did not influence tumor growth or intratumoral vasculature. However, tumor vasculature decreased after initiation of ganciclovir therapy in the HSVtk-transduced tumors ($p < 0.05$). Early patchy or diffuse necrotic changes associated with ultrasonographic evidence of scattered intratumoral hemorrhage occurred in tumors treated with ganciclovir. Reduction of the tumor blood supply may be an important feature of HSVtk transduction-mediated tumor regression and may, at least partially, account for the degree of tumor destruction that occurs despite the lack of transduction of all tumor cells.

KEY WORDS · **gene therapy** · **brain neoplasm** · **thymidine kinase** · **ganciclovir** · **tumor vasculature** · **bystander effect** · **rat**

THE ability to eradicate malignant brain tumors by selective retrovirally mediated transfer of the herpes simplex virus thymidine kinase (HSVtk) gene to tumor cells combined with ganciclovir therapy has recently been demonstrated in animal tumor models[1,6] and is now being evaluated in a clinical trial.[4] In animals, complete tumor regression was often achieved despite incomplete *in vivo* transduction of the tumor cells.[1,6] This phenomenon is believed to result from a "bystander" effect associated with the HSVtk/ganciclovir system and has been shown to occur both *in vitro* and *in vivo*.[1] The exact mechanism of such a bystander effect is poorly understood.

One component of this *in vivo* bystander effect may

be related to the observation that endothelial cells are also susceptible to retrovirally mediated gene transfer.[6,7,9] Endothelial cells of tumor blood vessels may integrate the retroviral vector and thus become sensitized to ganciclovir.[6,9] The proliferation of tumor vasculature and nearby host microvessels induced by the release of angiogenic factors[2] favors infection of endothelial cells and gene transfer by the retroviral vectors, which require active mitosis for gene integration and expression. Diffuse hemorrhages occurred during ganciclovir administration within the *in vivo* HSVtk-transduced tumor.[6] This suggests that tumor regression may be due, in part, to ischemia secondary to ganciclovir-induced destruction of endothelial cells that were infected with the retroviral vector and thus received the HSVtk gene.

We investigated the hypothesis that *in vivo* transduction with the HSVtk gene also targets tumor blood vessels in subcutaneously inoculated 9L gliosarcoma tumors in rats treated with intratumoral injections of HSVtk-producer cells and ganciclovir administration. Tumor growth and vasculature were evaluated with Doppler color ultrasound imaging, which permits the real-time display of high-resolution gray scale images of tissue simultaneously with a display of flow data from vessels within the scanned plane.[3,8]

Materials and Methods

Vectors and Cell Cultures

The HSVtk (G1TkSvNa.53) gene vector* has a G1 backbone derived from the Moloney murine leukemia virus. The vector contains the herpes simplex thymidine kinase gene just downstream of the 5' long terminal repeat, which it uses as its promoter. The simian virus-40 early promoter serves as an internal promoter for the neomycin phosphotransferase gene NeoR, which confers resistance to the neomycin analog G418. The vector is packaged by the amphotropic retroviral-vector producer cell line PAT 2.4, which is derived from NIH3T3 cells, and has a titer of 0.5×10^6 colony-forming units/ml on NIH3T3 cells. The cell line was negative for replication-competent virus by S+/L−assay.

The cloned vector-producer cell line was maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (50 U/ml), streptomycin (50 μg/ml), and Fungizone (amphotericin B, 2.5 μg/ml). The vector-producer cells were grown in T-175 flasks. The cells were harvested prior to intratumoral injection by incubation in 0.05% trypsin-ethylenediamine tetra-acetic acid for 5 to 10 minutes at 37°C. The cells were collected in Hanks' balanced salt solution (HBSS), washed twice, and resuspended at 6×10^7 cells/ml for injection.

The G1TkSvNa.53 vector was transferred *in vitro* into the rat 9L gliosarcoma cell line using supernatant from a confluent culture of producer line cells. The transduced cell lines were then selected in G418 medium (1.0 mg/ml) for 7 days and subsequently used for brain tumor induction.

Tumor Inoculation, Cell Injection, and Ganciclovir Therapy

The study was performed in accordance with the National Institutes of Health guidelines for the care of laboratory animals and was approved by the *Institute Animal Care and Use Committee*.

Brain Tumors. To evaluate the effect of *in vivo* transduction on tumor vasculature during ganciclovir therapy, 10 Fischer 344 rats, each weighing 230 to 350 gm, received stereotactic inoculation of 4×10^4 9L tumor cells mixed with 4×10^4 HSVtk vector-producer cells into the rat's right frontal lobe, as previously described.[6] Ten other rats received 4×10^4 9L tumor cells that had been pretransduced with the HSVtk gene and selected in G418 medium.[6] Seven days after tumor inoculation, ganciclovir was administered intraperitoneally to both groups (15 mg/kg/ml twice a day for 7 days). The animals were then sacrificed and the tumors were removed for histological examination.

Subcutaneous Tumors. Twenty Fischer 344 rats, each weighing 230 to 350 gm, were anesthetized using intraperitoneal ketamine (90 mg/kg) and xylazine (10 mg/kg). The rats' flanks were shaved and coated with betadine, and 1.0×10^6 9L cells in 100 μl of HBSS were injected subcutaneously into the flanks to induce bilateral tumor growth, for a total of 40 tumors. On Day 21 after inoculation, all tumors were injected with 10 intratumoral injections (100 μl each) of HSVtk vector-producer cells (6×10^7 cells/ml) using a No. 30 needle. Each injection was given over 1 minute. The injections were equally distributed over the circumference of the subcutaneous tumor. After 7 days, ganciclovir (15 mg/kg) was administered intraperitoneally to 10 HSVtk-injected rats (harboring 20 treated tumors) twice a day for 14 days. Ten rats served as controls and received saline injections (1 ml) instead of ganciclovir for 14 days.

Doppler Ultrasound Studies

Each subcutaneous tumor underwent ultrasound evaluation just before cell injection (Day 21 after inoculation), just before initiation of ganciclovir therapy (Day 28 after inoculation), during ganciclovir administration (Day 35 after inoculation), and after termination of ganciclovir treatment (Day 42 after inoculation). In keeping with the guidelines for animal care, the tumor burden in the control animals required termination of the study after ganciclovir administration was completed.

A single ultrasonographer, who was unaware of the treatment status of each rat, performed all studies and assessed the data. A color Doppler flow ultrasound imager† was used to visualize all tumors. A 7.5-MHz phased linear array transducer with axial and lateral image resolution of less than 1 mm was employed. The Doppler ultrasound parameters were as follows: the threshold was set just below noise level, ranging between 14 and 18 Hz; all studies were performed with the high-flow setting; and the gain was set to provide optimum image quality. The transducer was applied to the skin directly over the tumor to visualize it in the transverse and longitudinal planes. At each examination, the image was assessed for tumor vascularity (visible in color), gray scale echogenicity, and the presence or absence of fluid. Blood vessels were enumerated by scanning the tumor on the transverse and horizontal planes at 5-mm intervals. The highest number of vessels from either plane was recorded. An increase in echogenicity was considered evidence for hemorrhage or early necrosis, and stationary fluid (no Doppler ultrasound signal) appearing in the tumor was considered evidence of necrosis. From the transverse and longitudinal scans, the three dimensions

* Gene vector supplied by Genetic Therapy, Inc., Gaithersburg, Maryland.

† Angiodynograph ultrasound imager, Model 1, manufactured by Quantum Medical Systems, Issaquah, Washington.

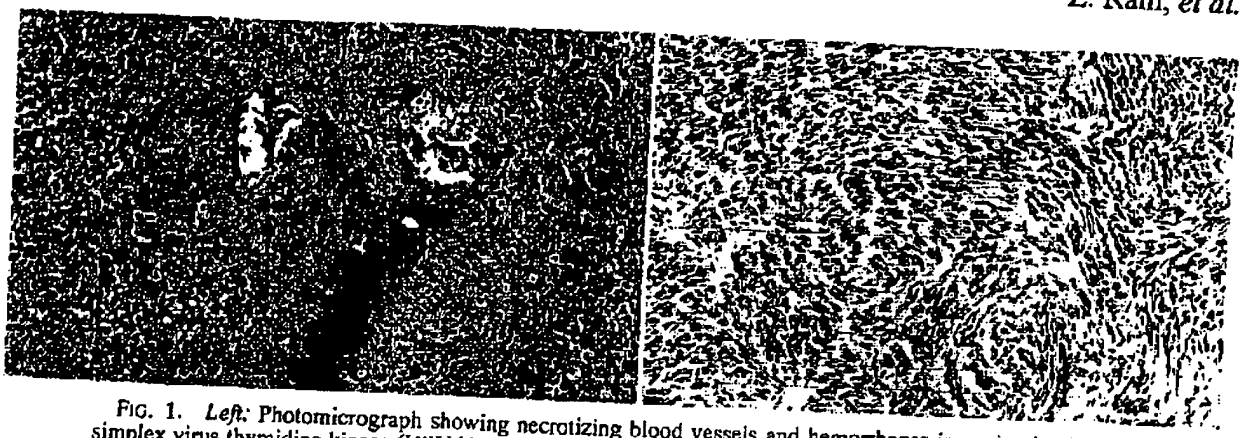

FIG. 1. *Left:* Photomicrograph showing necrotizing blood vessels and hemorrhages in an *in vivo* herpes simplex virus thymidine kinase (HSVtk) gene-transduced 9L brain tumor after 7 days of ganciclovir therapy: H & E, × 106. *Right:* Photomicrograph demonstrating no hemorrhage or vascular damage after 7 days of ganciclovir therapy in a 9L brain tumor pretransduced *in vitro* with the HSVtk gene before inoculation. H & E, × 106.

of the tumor, its thickness (T), width (W), and length (L) in cm, were measured directly on the screen using electronic calipers. A tumor volume was then calculated using the formula for a prolate ellipsoid: volume (cu cm) = (T × W × L) × 0.52.

Statistical Analysis

The paired t-test was used to compare values obtained from individual tumors at various phases of the experiment. The Wilcoxon signed-rank test was used to compare means between groups. The chi-squared test was used to analyze the frequency of necrotic changes within the tumors at various time points.

Results

Brain Tumors

Diffuse hemorrhages and necrosis were detected in rats inoculated with a mixture of wild-type 9L and HSVtk vector-producer cells. Necrotic blood vessels were occasionally present (Fig. 1 *left*). In the rats that were inoculated with tumor cells pretransduced with the HSVtk gene, no hemorrhages or abnormal blood vessels were detected although tumors were significantly smaller than controls, as previously described[6] (Fig. 1 *right*).

Blood Supply in Subcutaneous Tumors

The number of tumor blood vessels increased significantly over the 7-day period between producer cell injection and initiation of ganciclovir or saline treatment (2 ± 1.6 vs. 3 ± 1.2, mean ± standard deviation, $p < 0.05$). A significant decrease in the mean number of tumor blood vessels was observed in the ganciclovir-treated rats during ganciclovir administration (3.5 ± 1.2 to 0.8 ± 1.2 at 14 days after beginning ganciclovir therapy, $p < 0.05$) while during the same period, a significant increase occurred in the control tumors (2.55 ± 1.2 to 4.15 ± 1.7, $p < 0.05$). After ganciclovir treatment, a significant difference in the mean number of blood vessels was observed between the two groups ($p < 0.05$, Wilcoxon signed-rank test) (Fig. 2).

Subcutaneous Tumor Growth and Consistency

At the termination of the experiment (due to tumor burden in the control rats), there was no significant difference between the mean tumor volume of the two groups (5.1 ± 2.6 ml for treated tumors vs. 7.2 ± 5.2 ml for untreated tumors, $p = 0.25$). This type of tumor (9L glioma) tends to become necrotic over time. Evidence of tumor necrosis appeared earlier in the ganciclovir-treated rats than in the control animals: 7 days after initiation of ganciclovir therapy, 88% of the ganciclovir-treated tumors but only 28% of tumors in control rats had evidence of necrotic change on ultrasound studies ($p < 0.01$, chi-squared test, Fig. 3). In the ganciclovir-treated tumors, a hyperechoic signal, consistent with intratumoral hemorrhage, was observed in 25% of tumors on Day 7 of ganciclovir therapy and

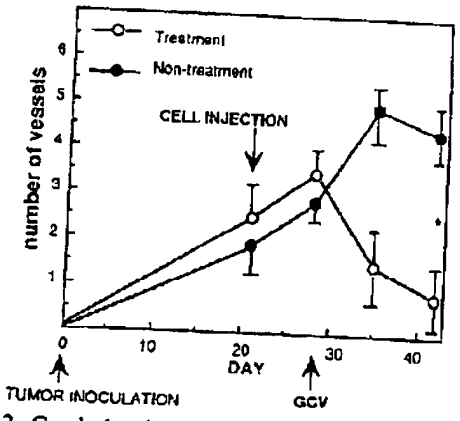

FIG. 2. Graph showing the number of blood vessels in subcutaneous tumors in rats as enumerated during Doppler ultrasound examination during the various phases of the study (means ± standard deviations). A significant reduction in the number of tumor blood vessels occurred after 14 days of ganciclovir (GCV) therapy *(asterisk)*. *Open circles* = rats treated with ganciclovir; *closed circles* = control animals receiving saline.

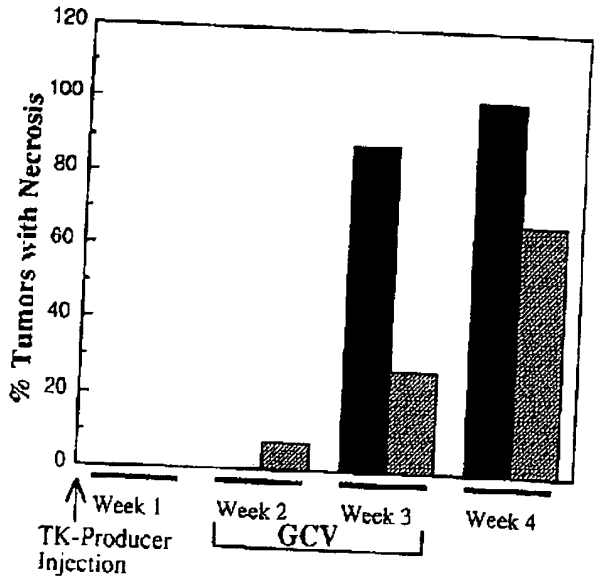

FIG. 3. Bar graph demonstrating the percent of subcutaneous tumors with necrosis detected by ultrasound at the different phases of the study: Week 1 = after producer cell injection; Week 2 = beginning of ganciclovir (GCV) or saline administration; Week 3 = 2nd week of ganciclovir or saline administration; and Week 4 = after treatment completed. *Black bars* = ganciclovir-treated tumors, *striped bars* = tumors in animals that received saline instead of ganciclovir. TK = thymidine kinase.

in 50% of tumors on Day 14 of ganciclovir therapy, while no hyperechoic signals were detected at either time point in the tumors in animals treated with saline. Figure 4 illustrates the typical ultrasonographic appearance of the ganciclovir-treated tumors at the various stages of the study.

Discussion

The mechanisms underlying complete tumor eradication by retrovirally mediated transfer of the thymidine kinase gene and ganciclovir therapy are not fully understood. A potent bystander effect probably contributes significantly to this process, since only a fraction of tumor cells incorporate the HSVtk gene and are rendered susceptible to the basic mechanism of interruption of deoxyribonucleic acid synthesis by thymidine kinase-dependent phosphorylation of ganciclovir. The bystander effects exist in both *in vitro* and *in vivo* models[1,6] and may comprise several independent mechanisms. Release of a phosphorylated form of ganciclovir, perhaps in a cyclic form, and transfer of other toxic by-products via intercellular channels (gap junctions) may play a role in this bystander effect (RM Blaese, unpublished data).

Tumor ischemia resulting from destruction of HSVtk-transduced endothelial cells by ganciclovir represents another potential bystander mechanism that may augment tumor eradication *in vivo*. Tumoral secretion of angiogenic factors induces endothelial mitogenesis and rapid proliferation of local blood vessels,[2,5] allowing retroviral integration and gene transfer into the endothelial cells of these angiogenic vessels to a greater extent than would be expected in the relatively quiescent nontumor blood vessels.[6,9] Diffuse hemorrhages appeared in HSVtk-transduced brain tumors in rats during the course of ganciclovir therapy when transduction was achieved *in vivo*, but not when the tumor had been transduced *in vitro*. This is due to the presence of viral vector particles capable of transducing endothelial cells only when vector-producer cells are present within the tumor (*in vivo* transduction). These observations caused us to hypothesize an ischemic mechanism as part of the process that leads

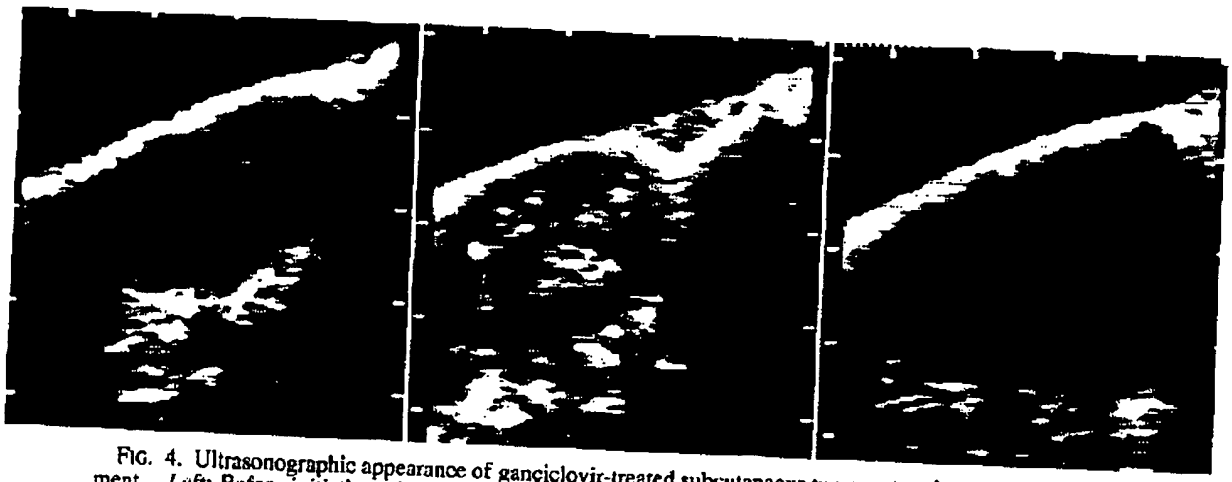

FIG. 4. Ultrasonographic appearance of ganciclovir-treated subcutaneous tumors at various phases of treatment. *Left:* Before initiation of ganciclovir therapy, the tumor is homogeneous. *Center:* Seven days after initiation of ganciclovir treatment, there are multiple intratumoral hyperechoic signals representing hemorrhage sites. *Right:* On Day 14 after initiation of ganciclovir administration, multiple necrotic cavities are evident within the tumor.

to tumor eradication during HSVtk-gene transfer therapy.

Our data suggest that, in the rat subcutaneous glioma model, tumor blood vessels were affected early and significantly after *in vivo* HSVtk-transduction and ganciclovir therapy. Although the observed changes in the tumors could arise as a consequence of tumor cell death, the fact that the ultrasonographic evidence of hemorrhage and necrosis in the tumors occurred before an effect on tumor size raises the possibility that they were secondary to direct vascular damage and ischemia. The results of these experiments suggest that reduction of the tumor blood supply is an important accompanying feature of HSVtk transduction-mediated tumor regression *in vivo* and may account, at least in part, for the tumor destruction that occurs despite limited gene transfer in the tumor.

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Address reprint requests to: Zvi Ram, M.D., Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Building 10–5D37, 9000 Rockville Pike, Bethesda, Maryland 20892.